Water Mediated Interaction between Phospholipid and Saccharide in Aqueous Solution and Frozen State

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The heating thermograms of differential scanning calorimetry (DSC) on the binary and three component systems, saccharide/water, dipalmitoylphosphatidylcholine (DPPC)/water and saccharide/DPPC/water, were investigated at various compositions from -120°C to 40°C . Maltose and glucose were used as saccharides. The thermograms of the saccharide/DPPC/water system were very similar to those of the saccharide/water system. The average amount of unfreezable water per molecule in the ternary system was found to be simply an arithmetic average of those of the binary systems. The solid-liquid state diagram of saccharide and water in the DPPC/saccharide/water system was analyzed. Based on these results, no direct interaction between the saccharide molecules and DPPC molecules in the frozen state was concluded. The saccharides were found to raise the gel-liquid-crystalline phase transition temperatures of the hydrated multilamellar of DPPC, and its mechanism was proposed to be a water-mediated interaction.

Keywords differential scanning calorimetry; saccharide; dipalmitoylphosphatidylcholine (DPPC); unfreezable water; phase transition; hydrogen-bonding; frozen state

Introduction

Recently, lipid vesicles constructed from bilayers of phospholipid molecules, so-called "liposomes," have been extensively studied as a potential drug carrier in the pharmaceutical field. In this connection, freezing^{1,2)} and freeze-drying^{3,4)} of liposomes have been attempted with the addition of stabilizers such as saccharides and polyalcohols. Many studies^{2,5,6)} have revealed the stabilizing mechanisms of saccharides. In the freezing state, two damage mechanisms of liposomes should be considered, *i.e.*, the mechanical breaking of the membrane caused by ice crystal formation, and the fusion of liposomes. In the saccharide solution, liposomes are covered with a concentrated solution or glass of the mixture of the saccharide and the unfreezable water in the freezing state, and it prevents liposomes from penetration by ice crystal and from fusion.²⁾ In the freeze-drying process, liposomes are exposed to a highly desiccated environment after the freezing. The damage of liposomes has been ascribed to both their fusion and the passing of the gel-liquid-crystalline transition during the rehydration process of the freeze-dried liposomes. This damage can be inhibited by the saccharide layer covering the liposomes by hydrogen-bonding with the phosphate groups of phospholipids in the dry state.^{4,6)} Such hydrogen-bonding was also found in the freeze-dried mixture of a water soluble protein and a saccharide,⁷⁾ and was reported to play an important role in the stabilization of the protein during freeze-drying.^{7,8)}

Although the formation of hydrogen-bonding is a well-accepted fact, it is still not clear when this bonding forms during the freeze-drying process. In the present study, differential scanning calorimetry (DSC) heating thermograms of saccharide/water, dipalmitoylphosphatidylcholine (DPPC)/water and saccharide/DPPC/water system were investigated from -120°C to 40°C with maltose and glucose as saccharides. The temperatures of the melting of ice and the glass transition and quantity of unfreezable water per molecule (N) of the ternary system were compared with those of the binary systems. The effect of saccharides on the gel-liquid-crystalline phase transition temperature of hydrated multilamellar of DPPC was also investigated. Based on these results, we showed that there is no

hydrogen-bonding formation between saccharides and DPPC both in the solution state and in the freezing state.

Experimental

Materials DPPC was supplied from Nihon Seika Co., Ltd., and its purity was more than 99%. The DPPC was desiccated at 95°C and at 0.1 Torr on P_2O_5 for 6 h before use. The residual water content of the dry DPPC was assumed to be less than 1%, as it showed no endothermic transition peak below 90°C .⁹⁾ Glucose and maltose were obtained from Wako Chemical Co., Ltd. Glucose was desiccated at 90°C and at 0.1 Torr on P_2O_5 for 6 h, and maltose $\cdot \text{H}_2\text{O}$ was desiccated at room temperature and at 0.1 Torr on P_2O_5 for 2–3 d before use. A molecule of the hydration water of maltose $\cdot \text{H}_2\text{O}$ is known not to evaporate by this desiccation process.¹⁰⁾ The mixtures of DPPC and saccharide were prepared by dissolving the appropriate amount of dry DPPC and dry saccharide in methanol-benzene (8:2) solution, and evaporating the solvent at 60°C and at 0.1 Torr for 1 h. The mixtures were then desiccated at room temperature and at 0.1 Torr on P_2O_5 for 2–3 d.

DSC Studies DSC thermograms were obtained by using a high sensitivity Seiko calorimeter (TC-100). Dry lipids, dry saccharides and dry mixtures were directly weighed in an aluminum or silver sample holder, respectively, except for the saccharide/water system of the saccharide weight percentage below 50%. The required amounts of water were added to the holders by a micro-syringe. After the sample holders were hermetically closed, they were equilibrated at 95°C for about 6 h. No water was lost during this procedure. The measurements were performed at a heating rate of $5^{\circ}\text{C}/\text{min}$ from -120°C to 40°C after they were cooled to -120°C at a cooling rate of about $10^{\circ}\text{C}/\text{min}$. For experimental convenience, only heating thermograms were obtained. The heat absorbed was determined from the area under the peaks using the heat of melting of ice as a standard. For measurement of the transition temperatures of hydrated DPPC, dry DPPC was directly weighed in silver sample holders. The required amounts of aqueous saccharide solution of various concentrations were added by micro-syringe. After being hermetically sealed, they were equilibrated at 55°C for longer than 2 h. The measurements were performed at $0.5^{\circ}\text{C}/\text{min}$ from 10°C to 70°C . Repeated heating runs produced the same thermal behavior.

Results

Saccharide/Water System The DSC heating thermograms of saccharide/water systems are obtained by changing the weight percentage of the saccharide. Figure 1 shows those of a maltose/water system and they represent several patterns depending on the maltose weight percentage (W_{mal}). At W_{mal} below about 10%, only an endothermic peak due to the melting of an ice crystal appeared: there is no peak due to the melting of the eutectic mixture. At W_{mal} above about 10%, a small step in the base line

Fig. 1. DSC Heating Thermograms of the Maltose/Water System
Weight percentage of maltose, a) 19%; b) 48%; c) 67%; d) 87%.

Fig. 2. The Solid-Liquid State Diagram of the Maltose/Water System
●, the peak temperature of melting of ice, (T_m); ■, the peak temperature of recrystallization, (T_r); ▲, the glass transition temperature, (T_g); ○, T_g' indicated in this test.

is detected near the starting temperature of melting. This may be due to the glass transition of the mixture of maltose and unfreezable water in the presence of ice crystal. In the range of 60% to 70%, glass transition is clearly observed, followed by recrystallization and melting of ice crystals. At W_{mal} above about 70%, only glass transition is exhibited. Based on the thermal data in Fig. 1, the solid-liquid state diagram can be obtained as shown in Fig. 2. T_m and T_r represent the peak temperatures of endothermic and exothermic peaks, respectively. T_g shows the glass transition temperature. T_g' corresponds to the temperature shown as a small step in Fig. 1b. The glucose/water system shows results similar to the maltose/water system. Franks¹¹⁾ reported the thermal data of the glucose/water system and combined them with other literature data in a solid-liquid state diagram.¹²⁾ Our results of the glucose/water system show good agreement with theirs, except for two points: transition temperatures are slightly different, and the two minor endothermic peaks detected by Franks *et al.*¹¹⁾ are not found in our data. These are probably due to differences in experimental conditions for thermal analysis. In Fig. 3, the relationship between the heat of melting per gram of the maltose/water system (H_f/g) and W_{mal} is represented. It shows a nonlinear

Fig. 3. The Relationship between the Heat of Melting of Ice per Gram of the Maltose/Water System (H_f/g) and the Weight Percentage of Maltose (W_{mal})

Fig. 4. DSC Heating Thermograms of the DPPC/Water System
Weight percentage of DPPC, a) 20%; b) 80%; c) 86%.

relationship, and the amount of unfreezable water per maltose molecule can be calculated from the composition at which H_f/g becomes zero. The values for glucose and maltose are 4.5 ± 0.4 and 6.7 ± 0.3 , respectively. They agreed fairly well with those from DSC by other workers.¹³⁾

DPPC/Water System The DSC heating thermograms of DPPC/water systems are investigated at various weight percentages of DPPC (W_{DPPC}) as shown in Fig. 4. They show good agreement with the thermograms reported by Grabielle *et al.*¹⁴⁾ As they analyzed in detail, at W_{DPPC} below 80%, the T_m hardly change and are around 0°C. When the W_{DPPC} is above 80%, the T_m begin to drop abruptly. No endothermic peak due to the melting of ice crystal is observed at W_{DPPC} above 87%. The quantity of unfreezable water can be calculated as 5.3 ± 0.5 molecules per molecule of DPPC.

The hydration properties of phospholipids have been already investigated by calorimetric measurements,¹⁴⁾ the adsorption isotherm method,¹⁵⁾ nuclear magnetic resonance (NMR)¹⁶⁾ and X-ray studies.¹⁷⁾ These studies have demonstrated the presence of several types of water with properties differing from the bulk state in the phospholipid/water system. Water molecules adjacent to the phosphate groups were most strongly bound with highly restricted

Fig. 5. DSC Heating Thermograms of the Maltose/DPPC/Water System at the Molar Ratio of Maltose to DPPC at Unity

Weight percentage of the mixture of maltose and DPPC (W_{mix}), a) 74.9%; b) 81.0%; c) 82.7%.

Fig. 6a. The Relationships between H_t/g and W_{mix} of the Glucose/DPPC/Water System

The molar ratio of glucose and DPPC, $\circ$, 1:1; $\bullet$, 4:1.

Fig. 6b. The Relationships between H_t/g and W_{mix} of the Maltose/DPPC/Water System

The molar ratio of maltose and DPPC, $\circ$, 1:1; $\bullet$, 7:3.

motion, and their amount was 4–6 molecules per molecule of phospholipid. In all probability, about 5 molecules of unfreezable water obtained in our experiment are assigned to this strongly bound water around the phosphate groups.

Saccharide/DPPC/Water System Figure 5 shows the DSC heating thermograms of the maltose/DPPC/water system at various weight percentages of the mixture of maltose and DPPC (W_{mix}). The molar ratio of maltose to DPPC is kept constant at unity. They produce thermograms similar to the maltose/water system (Fig. 1), suggesting that maltose molecules are fully hydrated. DSC heating thermograms are also investigated at various molar ratios of maltose or glucose to DPPC. These results give the relations between H_t/g and W_{mix} , and are obtained as shown in Fig. 6. The average quantities of unfreezable water (N) are calculated and shown in Fig. 7 as a relationship between N and the molar fraction of a saccharide (X). N are found to be linearly related to X in both systems. This result indicates that the average values of N for the saccharide/DPPC/water system are simply arithmetic averages of those for the saccharide/water and DPPC/water systems. It is suggested that there is no hydrogen-bonding formation between the phosphate group of DPPC and the $-\text{OH}$ group of a saccharide. Hydrogen-bonding sites of

Fig. 7. The Relationships between N and the Molar Fraction of Saccharide (X)

$\circ$, Glucose; $\bullet$, maltose. N : the average amount of unfreezable water.

TABLE I. Values of T_m , T_g , f_m and f_g at Various W_{mix} for Each Saccharide/DPPC/Water System

	Mole ratio	W_{mix} (%)	T_m (°C)	T_g (°C)	f_m	f_g
Glu : DPPC	1:1	65.4	-5.4	ND	5.5	ND
		80.7	-17.3	-85.8	4.6	5.3
		82.2	-20.8	-83.9	4.6	5.3
	4:1	73.8	-18.9	-83.7	4.3	4.6
Mal : DPPC	1:1	75.0	-20.5	-82.2	4.3	4.6
		74.9	-10.4	ND	5.6	ND
		81.0	-13.7	-67.2	4.6	5.0
	7:3	82.7	ND	-60.9	ND	5.0
		53.1	-4.3	ND	5.6	ND
		69.8	-11.3	ND	6.1	ND

ND = not determined.

the DPPC molecule for water molecules and saccharide molecules are considered to be same, *i.e.*, the phosphate groups of DPPC.⁶⁾ If there were direct interaction between DPPC and the saccharide, the N vs. X plot should deviate from a linear relationship.

Further evidence for the absence of direct bonding between saccharides and DPPC can be obtained from analysis of the transition temperature, T_m and T_g : assuming that there is no direct interaction between DPPC and saccharide, the liquid-solid phase diagram of saccharide and water in the DPPC/saccharide/water system must be identical to the diagram of the saccharide/water system. The weight percentage of saccharides comparable to the binary system (W'_s) can be represented by equation (1) by taking the hydration water of DPPC into account,

$$W'_s = 100 W_s / (W_s + W_h - f M_{\text{H}_2\text{O}} W_D / M_D) \quad (1)$$

where W_s , W_h and W_D are the weight percentages of saccharide, water and DPPC in the saccharide/DPPC/water system, respectively. $M_{\text{H}_2\text{O}}$ and M_D are molecular weights of water and DPPC. The f is the hydration number of water molecules per a molecule of DPPC. The values of W'_s are obtained from the binary diagram for each experimental value of T_m or T_g in the ternary system, and f values are calculated by Eq. 1. The f_m and f_g correspond to T_m and T_g , respectively. The values are summarized in Table I. As shown in Table I, the average value of f is about 5, and it shows good agreement with the quantity of water which can form hydrogen-bonding with the phosphate group of DPPC as mentioned above. The result well confirms the

Fig. 8a. The Relationships between the Transition Temperatures and the Maltose Concentration

●, T_c ; ○, T_p .

Fig. 8b. The Relationships between the Transition Temperatures and W_{DPPC}

●, T_c ; ○, T_p .

Fig. 9. The Relationship between the Increment of T_c , ΔT , and [Maltose]/[DPPC] at Two Maltose Concentrations

●, 20%; ○, 32%.

validity of the assumption that there is no hydrogen-bonding formation between the phosphate groups of DPPC and a saccharide molecule during the freezing state. They interact most probably through hydrogen bonded water molecules.

Effect of Saccharides on the Transition Temperature of Hydrated DPPC DSC heating thermograms of the hydrated multilamellas of DPPC in a maltose or glucose aqueous solution are investigated at various saccharide and DPPC concentrations. Figure 8a shows the transition temperatures of DPPC, i.e., the main transition temperature of $P'_\beta \rightleftharpoons L_\alpha$ (T_c) and the pre-transition temperature of $L'_\beta \rightleftharpoons P'_\beta$ (T_p) as a function of the maltose concentration, [maltose], at the DPPC concentration, [DPPC], of 1%. Both T_c and T_p rise with the maltose concentration, and at a maltose concentration of 50%, T_c and T_p are 1.5°C and 4°C higher than in the absence of maltose, respectively. Enthalpy change, ΔH , of each transition does not depend on [maltose]. The relationships between the transition temperatures and the glucose concentration are similar to those for maltose. The increment of T_c , ΔT , is shown in Fig. 9 as a function of the ratio of [maltose] to [DPPC], [maltose]/[DPPC], at [maltose] of 20% and 32%. These

results show that ΔT is not dependent on [maltose]/[DPPC] but on [maltose].

Some saccharides are known to increase the phase transition temperatures of the hydrated multilamellas of DPPC.^{5,6,18} Strauss *et al.*⁵) found such increases in a sucrose aqueous solution, and attributed their origin to a direct interaction between the sucrose molecule and DPPC molecule. If the increase were caused by such direct interaction as they mentioned, ΔT should depend not only on [maltose] but also on [maltose]/[DPPC]. Our results show that ΔT scarcely depends on [maltose]/[DPPC]. Therefore, no direct interaction may exist between the maltose molecule and the DPPC molecule in the presence of bulk water in this system. The next question is, "Why the transition temperatures of the hydrated multilamellar of DPPC increase with the saccharide concentration?" It is reported that T_c and T_p in the DPPC/water system increase by decreasing the weight percentage of water, W_{H_2O} , below 25%, because the distance between the bimolecular membrane of DPPC becomes shorter and the ordering degree of the water increases; in other words, the hydration state of the head group of DPPC changes.^{19,20} In Fig. 8b, the relationships between T_c or T_p and W_{DPPC} for the DPPC/water system are represented from the data published by Grabielle *et al.*¹⁹) The increasing behavior of the transition temperatures in Fig. 8a is very similar to that in Fig. 8b. The reason for the increase observed in our results can be ascribed to the change in the hydration state of the head groups of DPPC, that is, the water mediated interaction.

Discussion

Although it is well established that the formation of hydrogen-bonding between a saccharide and phospholipid molecules is indispensable for the stabilization of liposomes during freeze-drying, it is not clear at which stage this bonding forms during the freeze-drying process. Strauss *et al.*⁵) showed that the stabilization ability of sucrose was abolished by the addition of Europium (Eu^{3+}), which forms an ionic linkage, and also that sucrose increased the transition temperatures of hydrated multilamellar vesicles. From these results they suggested that the direct interaction between saccharides and phospholipids occur in the presence of bulk water. On the contrary, Crowe *et al.*⁶) and Rudolph *et al.*²⁰) proposed that the interaction likely occurred during freezing when the phospholipids and saccharides were concentrated well beyond their bulk concentration. They mentioned that the increase of the transition temperature of hydrated multilamellar by the addition of saccharides was due to an osmotic dehydration of the polar head groups, because they could not detect a T_c -elevating effect on unilamellar vesicles. They showed that "Eu³⁺-induced" fusion during the freeze-thawing of a liposome suspension was reduced relative to an increasing saccharide concentration. From this result they suggested that there was competition for bond-formation for the head group of DPPC between the saccharide and the Eu^{3+} in a freezing state.²¹)

Our results show that amount of strongly bound water of the phosphate groups of DPPC is not changed by the addition of saccharides in a freezing state. And the elevation of T_c or T_p of hydrated DPPC by the addition of a saccharide

is ascribed to a water mediated interaction. From these results, the formation of hydrogen-bonding most likely occurs after the removal of a large amount of water from the system during freeze-drying. Based on our interpretation, we suspect that the reduction of "Eu³⁺-induced" fusion of liposomes by the addition of saccharides observed by Anchordoguy *et al.*²¹⁾ is due to the high viscosity of the system. Because the fusion follows the collision of liposomes, the degree of the fusion is greatly affected by the viscosity near the liposome. In a frozen state, the surface of the liposome is covered with a concentrated aqueous or glassy saccharide layer, whose viscosity is very high. In such situations, Eu³⁺-induced fusion is most probably prevented by an increase in saccharide concentration.

In conclusion, we found that there is no direct interaction between the saccharide and phospholipid molecules in the aqueous solution state or in the frozen state.

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